AD_____

Award Number: W81XWH-10-1-0372

TITLE: Microfluidic Flow Retardation for Tagless Cancer Cell Analysis

PRINCIPAL INVESTIGATOR: Robert Wieder

CONTRACTING ORGANIZATION: UMDNJ-New Jersey Medical School
Newark, NJ 07103

REPORT DATE: July 2012

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
July 2012	Final	1 July 2010 - 30 June2012

4. TITLE AND SUBTITLE

Microfluidic Flow Retardation for Tagless Cancer Cell Analysis

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-10-1-0372

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Robert Wieder

E-Mail: wiederro@umdnj.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

UMDNJ-New Jersey Medical School
Newark, NJ 07103

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

We developed a microfluidic device to detect surface protein expression in individual cancer cells in small cell populations without prior labeling. We used LM2 cells (kind gift of Juan Massague, Memorial Sloan-Kettering Cancer Center, NY), derived from murine lung metastases of MDA-MB-231 cells with a unique lung metastatic potential. We selected LM2 cells for 100% sustained expression of IL-13RA2 and cells without expression. We coated the channel with an antibody to IL-13RA2 or with BSA and directed cells over the patch by a microsyringe pump at flow rates of nanoliters per second. The transient interactions with surface ligands resulted in cell flow retardation in cells expressing the cognate surface protein over the antibody-coated patches and rapid, unimpeded flow on BSA or in the absence of the antigen with 100% sensitivity and 100% specificity.
We detected and tracked cells with microscope objectives and computer tracking programs. We were able to multiplex 6 surface proteins creating a barcode for their expression in each individual cell in a population. We characterized cells in mixed tumors using needle aspirates. All objectives were achieved.

15. SUBJECT TERMS

Microfluidics, cell flow retardation, transient bond formation, surface protein detection

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	15	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

INTRODUCTION:

Solid tumors are highly heterogeneous with respect to cell type. A small fraction of cells with the capacity to metastasize will dictate the ultimate prognosis of a tumor. Metastatic potential is dictated to a great extent by the expression pattern of cell surface proteins. Population averaged gene expression profiling by microarrays does not capture the signature of this minority subpopulation and therefore lacks the prognostic specificity to impact clinical practice. Needle aspirates of tumors reveal only a malignant phenotype in the few cells that are obtained and there are currently no good methods for characterizing cells when only a few are available. To address this need, we undertook to develop a microfluidic device to detect the surface protein expression profile of individual cancer cells without prior labeling. The device will be a microfluidic channel with multiple tandem antibody-coated patches over which cancer cells are directed by a microsyringe pump at flow rates of nanoliters per second. The transient interactions with surface ligands result in retardation of cell velocities over patches with cognate ligands of expressed surface proteins and normal cell velocities over patches coated with ligands that don't recognize surface proteins expressed by the cell. This will permit assigning an individual expression pattern for the proteins recognized by the immobilized antibodies for each cell. We hypothesize that the use of this device will make it possible to identify rare cells in a tumor with specific capacities to metastasize to unique organs based on their cell surface expression profiles with sensitivities and specificities of greater than 99%.

BODY:

Task 1. To Isolate and define breast cancer and tumor matrix-associated cell populations

We decided to characterize surface proteins associated with metastatic potential of breast cancer cells to the lung. We are using LM2 cells (kind gift of Juan Massague, Memorial Sloan-Kettering Cancer Center, NY) (1), derived from murine lung metastases of MDA-MB-231 cells that have a unique potential to re-metastasize to lung. We have verified that of the genes with differential expression patterns between LM2 and parental cells on gene arrays, four coded for surface proteins whose protein expression also differed between the two populations. We determined that the surface expression of VCAM, IL13RA2 and EMP1 was upregulated and that of CXCR4 was downregulated in LM2 cells as compared to MDA-MB-231 cells. We sorted the cells into three fractions for no, medium and high expression of these proteins. Figure 1 demonstrates that the majority of LM2 cells express high levels of IL-13RA2 and that a sorted population, of which 100% of cells express this surface protein, maintain expression after several weeks of passage. Cells sorted for lack of expression of CXCR4 or for expression of VCAM and EMP1 did not maintain the level of expression of the sorted cells after two weeks in culture and hence, those antigens could not be used for development of the parameters of microfluidic retardation. Thus, all of the studies described were performed with the cells with sustained expression of IL13R and with non-expressing control LM2 P1, MDA-MB-231 and MCF-7 control cells.

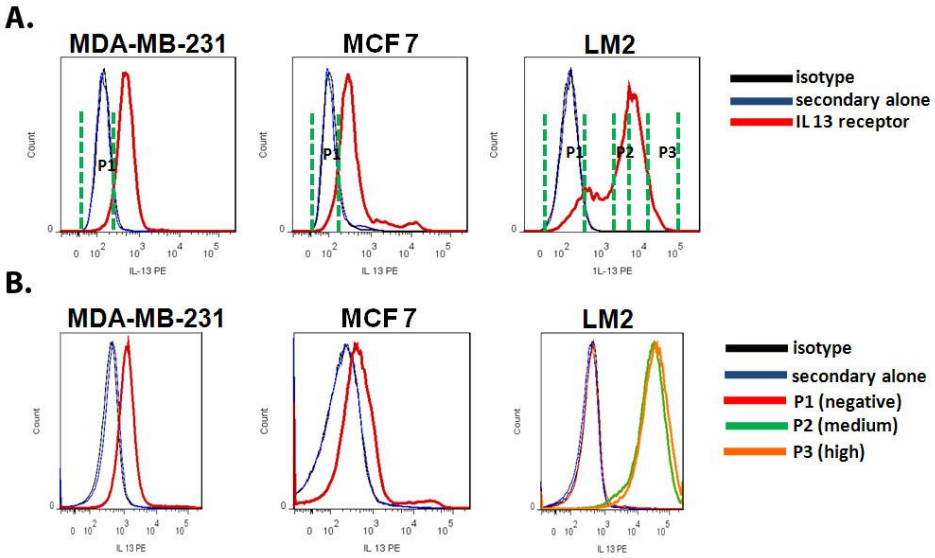

Figure 1. A. Sterile sorting of LM2 cells (P1, P2, P3), MDA-MB-231 and MCF-7 cells and maintenance of high levels of IL13 receptor (IL13R) expression in culture. A. LM2 cells expressed a significant fraction of cells with high expression of IL13R of >2 logs over other cells. Cells were sorted for low, medium and high expression and passaged for 2 weeks. B. Analysis of sorted and passaged cells demonstrated that P2 and P3 cells maintained a homogeneous, approximately 200x higher expression of IL13R than P1 or the other two cell lines. Scatchard analysis is being conducted to correlate these measurements with receptor numbers.

Task 2. To design, fabricate and calibrate a 14 patch impedance-monitored flow retardation device with visual cell identification with data recording and handling capability

We constructed a flow channel coated with IL13R antibody and BSA and conducted a series of experiments with different shear rates due to variable channel dimension, flow rates and viscosity from variable glycerol concentrations. The device (Figure 2) consisted of apposable glass plates with an inlet port for buffer, an inlet port for cells 5 mm downstream, and an outlet port for collection of buffer and cells. Tandem patches for BSA and IL-13RA2 antibody immobilization were separated by spacers of variable widths. Channel height was established by variable sized gaskets. We developed and characterized quantitative antibody adhesion to a microfluidic channel. More than 50% of adhered antibody remained on the patch after extensive washing with buffer pumped through the channel.

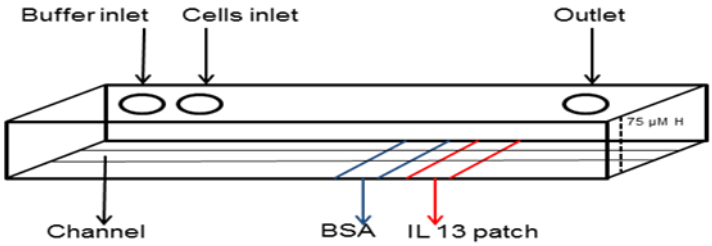

Figure 2. Shown is a sketch of the flow device used. Patches of immobilized ligands are 0.5 cm in length, but recent variations that provided reproducible flow rates include 100-200 micron patches with 100 micron spacers. Chamber heights varied from 25-100 μm depending on gaskets used to separate plates and provided variations in shear rate.

Cells are suspended in standard media at approximately 100,000 cells/ml. This is sufficiently dilute to permit sufficient distance between single file cells to prevent interference with

each other's flow. Volumes of 3.5 ml drawn up into a syringe are loaded onto the Harvard Apparatus Pump II syringe pump controller and pumped through Teflon tubing into the cell inlet port of the microfluidic channel in single file. Equal flow rate of buffer solution containing DMEM + 2.0 mM EDTA is pumped into the Buffer inlet. Flow rates are modulated in the 5-15 microliters/min range, determined in calibration experiments to establish a consensus shear rate, as before (2). Individual cells with varying phenotypes tracked by imaging will be collected from the outflow channel into different tubes for further analysis, initially manually. The current detection and tracking of cells with microscope objectives and computer tracking programs and measurement of the velocity of individual cells as they traverse individually coated patches is sketched below (Figure 3).

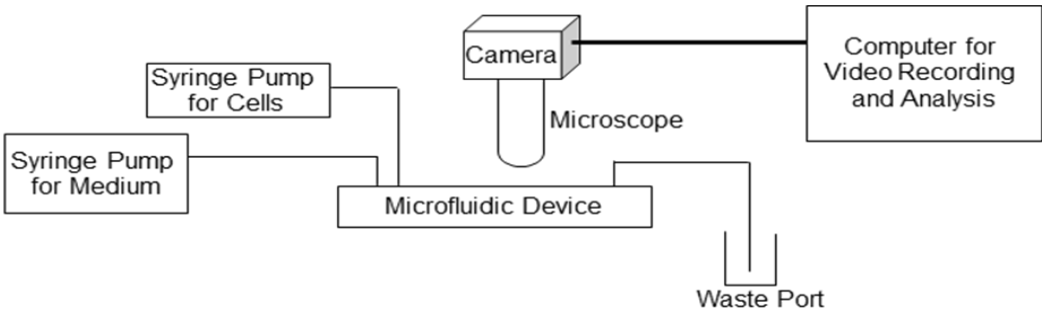

Figure 3. Current setup of the microfluidic device, pumping system and imaging and computational support. The waste port has the capacity to recapture specific cells for further analysis.

One of the major challenges is imaging a large protein-coated region while maintaining a high resolution that contains enough detail to allow tracking of cells over time. We use a latest generation high-resolution camera, an Epix Silicon Video 642 Camera with PIXCI Digital Frame Grabber attached to an inverted microscope with a 2X lens.

Interval timing is set to 2 images per second using a remote control cable. Images are recorded for 12 minutes on internal memory. Format used for recording images is high quality JPEG that allows facile import of images into image analysis software. Each series of images is imported into XCAP v3.2 software (Epix Inc., Buffalo Grove, IL). XCAP software permits tracking of each cell in successive images. An image containing no cells is subtracted from all images to remove all background noise. Cells are identified using 'blob' finding algorithm, which identifies all features with diameters between 5 and 15 pixels. Since cells are projected at 10 pixels each, they are readily identified. 'Tracking' algorithm identifies tracks of the cells and calculates velocity of cells (delta H) by measuring distance traveled in a half second. The data is imported into Microsoft Excel and percentage retardation will be calculated.

We demonstrated statistically significant shifts in mean flow rates in sorted populations of IL13R (figure 4, D&E) on IL13R antibody. The standard deviations of the flow rate distributions across cognate antibody patches were much higher than those over control patches. They were restored with blocking antibody (Figure 4, F&G). The reason for the wider distribution curve was the significant rate of retardation in about 20% of the cells, which had cell velocities more than two standard deviations lower than the mean velocity of the cells on control patches in the experiment shown in figure 6, where the chamber height was 50 μm and flow velocity was 15 μl/min, giving a shear rate of 100/s with a glycerol concentration of 1.5%. The velocity of the remaining 80% of the cells fell within the distribution curve of the velocity of the control patches, despite having a uniformly high expression of IL13R. This rendered the specificity greater than 99% but the sensitivity, using this measurement, only about 26% (Figure 4).

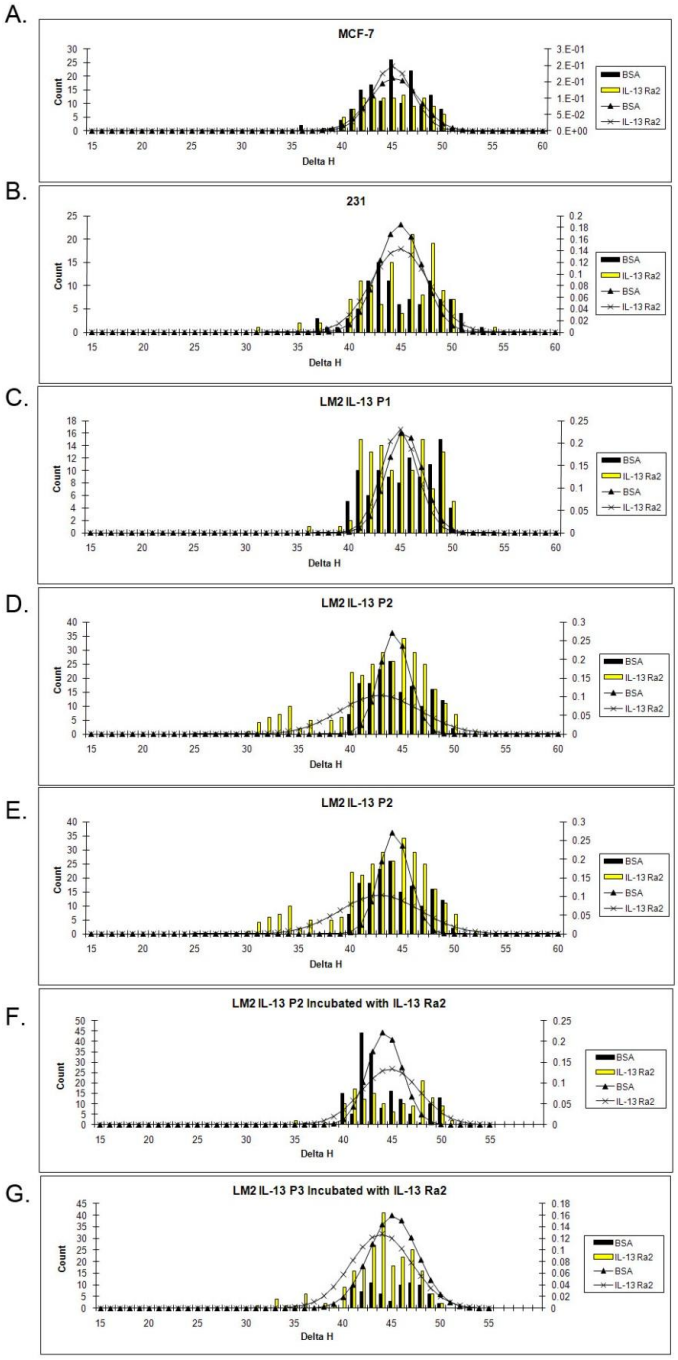

Figure 4. Sample flow retardation experiment with immobilized anti-IL13 receptor antibody. Below is the sketch of the flow device used. Table1 delineates the results demonstrating the percentage differences in the mean flow rates correlating with the presence of IL13R expression in sorted cells in this experiment. More significantly, the raw data depicted in figures 4 A-G demonstrate flow rates, depicted as differences in the number of pixels between cells (ΔH) in photographic frames taken at 0.5 second intervals. Only a subset of LM2 P3 and P3 cells had ΔH values below 35 (D & E), rendering specificity at greater than 99%, Sensitivity was around 26%. Blocking antibodies to IL13R eliminated flow retardation of LM2 P2 and P3 cells (F&G).

Table 1. Flow retardation experiment data from figure 4 conducted at conditions where chamber height was 50 μm and flow velocity was 15 μl/min (shear rate of 100/s) with a glycerol concentration of 1.5%.

Cell Line	Device Coating	Average	St. Dev.	%CV	%Decrease	P value
MCF-7	BSA	48.359	3.821	8%	0.5%	0.15423
	IL-13	48.140	3.081	6%		
231	BSA	47.912	5.489	11%	1.5%	0.34076
	IL-13	47.204	8.839	19%		
LM2 IL-13 P1	BSA	48.968	5.174	11%	1.6%	0.14377
	IL-13	48.161	3.786	8%		
LM2 IL-13 P2	BSA	47.271	4.740	10%	2.8%	0.09254
	IL-13	45.925	6.555	14%		
LM2 IL-13 P3	BSA	48.322	5.073	10%	11.4%	0.00009
	IL-13	42.828	3.596	8%		

The sensitivity improved somewhat to ~34% and specificity remained greater than 99% with chamber height of 50 μm, flow velocity of 9 μl/min (shear rate of 60/s) at a glycerol concentration of 0.75%. Decreasing the height of the chamber to 25 μm did not have a significant impact on further increasing sensitivity of specific interactions and retardation, even at slightly lower flow velocities. This was likely due to significantly increased shear rates negating any effect of increased cell-substratum contact compared to that in chambers of greater channel height.

By reanalyzing the data for rates in a 0.5 mm patch, we considered specific interactions as any that were more than two standard deviations below the average rates for all cells over control patches (Table 2). This provided a 100% specificity and a similar sensitivity at the 2 standard deviation cutoff, as did data when all runs across a patch were averaged for each cell. However, specificity remained 100% at lower differences than 2 standard deviations, while the sensitivity continuously increases to a maximum of 55%. If the cutoff was raised to around 1 standard deviation, the specificity dropped to 80% but the sensitivity rose to 71%. These analyses demonstrate that the unconsidered impulse to simply average rates over a patch will not necessarily differentiate between cells with specific and nonspecific binding with complete sensitivity and specificity.

Table 2. Reanalysis of data from the experiment shown demonstrating reciprocal relationship between sensitivity and specificity during analysis of the data.

Cutoff	Sensitivity	Specificity
40	32%	100%
41	35%	100%
42	45%	100%
43	55%	100%
44	55%	94%
45	71%	80%
46	81%	65%
47	94%	51%
48	97%	49%
49	97%	43%
50	100%	37%

Control experiments with patches coated with goat IgG instead of specific antibody demonstrated a complete lack of retardation of either P2 or P3 cells. Incubating cells with 250 ng/ml ILR13α2

antibody prior to the run completely eliminated flow retardation of LM2-P3 cells and LM2-P2 cells. Control preincubations with homotypic IgG did not have an effect on flow retardations of P3 cells or P2 cells. Incubating LM2-P1 cells with either IL13Rα2 antibody or homotypic pooled IgG did not have any effect on their cell velocities. The changes in mean ΔH values with blocking antibody demonstrate that retardation was due to specific interactions between IL13Rα2 and its cognate antibody.

To address the relatively low sensitivity of the system when comparing mean cell velocities in two entire populations, we considered the actual events that contributed to the results. Even in experiments where data capture was conducted at 50 msec. intervals, a fraction of the observations of a cell traversing a patch with a cognate antibody were of the cell traveling with the flow of the fluid above the patch (Figure 5A). Only some of the time did the cell descend to be in contact with the patch and was subject to flow retardation due to the transient bond formation. These measurement intervals were significantly slower than the averaged data. Hence, inclusion of 50 millisecond velocity measurements during spans of laminar flow with no interaction events factitiously raised the average velocity and detracted from the sensitivity of the assay.

These results led us to reanalyze the averaged data in Figure 5A and compare the distribution of cell velocities using only the slowest 50 millisecond measurement. Figure 5B depicts the distribution of cell velocities from Figure 5A when only the minimum measured 50 millisecond velocity was considered over each patch. The data show that P2 cells were distinguishable with 65.5% sensitivity and 100% specificity, with the test having a 100% positive predictive value and 75.9% negative predictive value when compared with the P1 cells. With P3 cells, all of which express IL13Rα2, the sensitivity, specificity, positive and negative predictive values were all 100%. We had configured the device for tracking and measuring cell velocities at 50 msec intervals as the same cell travels over two patches as proof of principle of multiplexing. Figure 5C demonstrates the delta H values of an LM2-P3 cell as it traverses a BSA-coated and an IL13Rα2 antibody-coated patch. The data clearly demonstrate that low delta H values are only present over the IL13Rα2 antibody-coated patch.

We reanalyzed flow data from individual cells taken at 50 microsecond intervals in the three LM2 selected subpopulations and in MDA-MB-231 cells under favorable conditions to include a broader representation of the cells passaged through the channel. Plotting the aggregate flow rates of LM2-P3 cells and P1 cells, it became apparent that the lower quartile of LM2-P3 cells always had flow rates on anti-IL13Rα2-coated patches that were slower and non-overlapping with flow rates on BSA-coated patches. We therefore plotted the lower quartile of flow rates in each observation to capture the true velocities during periods when the cells were interacting with the substratum. Figure 6 demonstrates the distribution of mean flow rates of the lower quartile in an experiment at a favorable shear rate and force in a 25 micron-high chamber, 10 microliter/minute flow rate in buffer containing 1.25% glycerol. The flow rates of LM2-P3 cells on anti-IL13Rα2-coated patches separate from those on BSA-coated patches 100% of the time while rates on the two patches overlap with MDA-MB-231 and LM2-P1 cells. LM2-P2 cells have distinct rates 93% of the time if a delta H of 1.27 (4 standard deviations below the BSA mean) is used as a cutoff, corresponding roughly to the percentage of cells positive for IL13Rα2 by flow cytometry. These data provide a more representative and convincing argument that the sensitivity and specificity of the assay is near 100%.

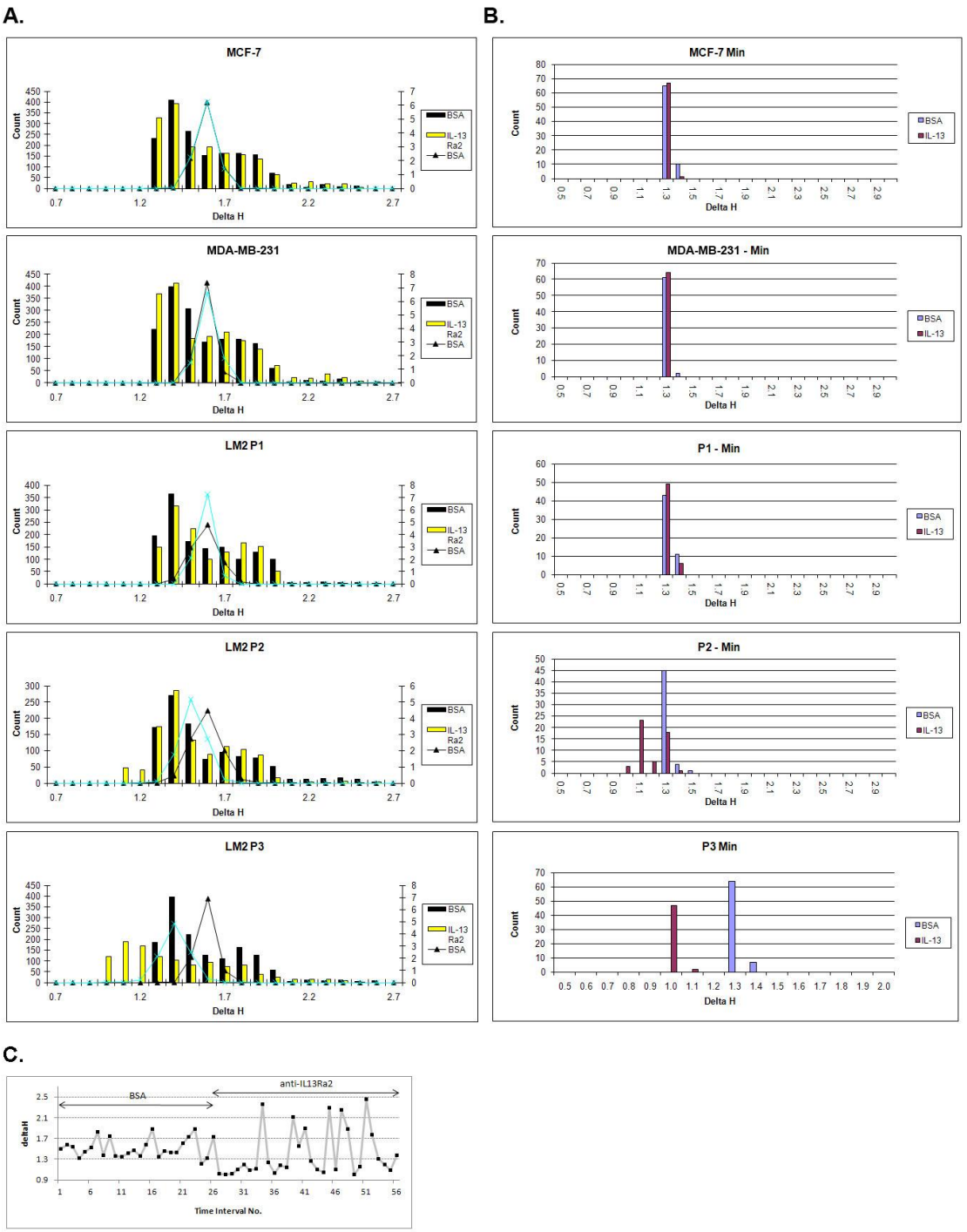

Figure 5. Complete data of all of the measured individual cell velocities collected over 50 msec intervals as each cell is observed traversing a BSA-coated and an IL13Rα2-coated patch under optimum shear rates at 25 μm chamber height, 1.25% glycerol and a total flow rate of 10 μl/min. **(**A) All of the ΔH values measured from each cell at 50 msec intervals over each patch plotted against observation frequency in all 5 cell lines. (B) Minimum ΔH values of the same cells graphed in A. as they are measured at 50 millisecond intervals over the two patches. Data demonstrate significant specificity in retardation of P2 and P3 cells in pooled ΔH analyses and near complete (P2 cells) and complete (P3 cells) separation of velocities over the two patches when minimum ΔH values were compared. C. Velocity measurements in a multiplexed device demonstrating the passage of the same LM2-P3 cell over two patches, one coated with BSA and one coated with IL13Rα2 antibody with delta H values measured at 50 msec intervals. The data clearly demonstrate that low delta H values are only present over the IL13Rα2 antibody-coated patch.

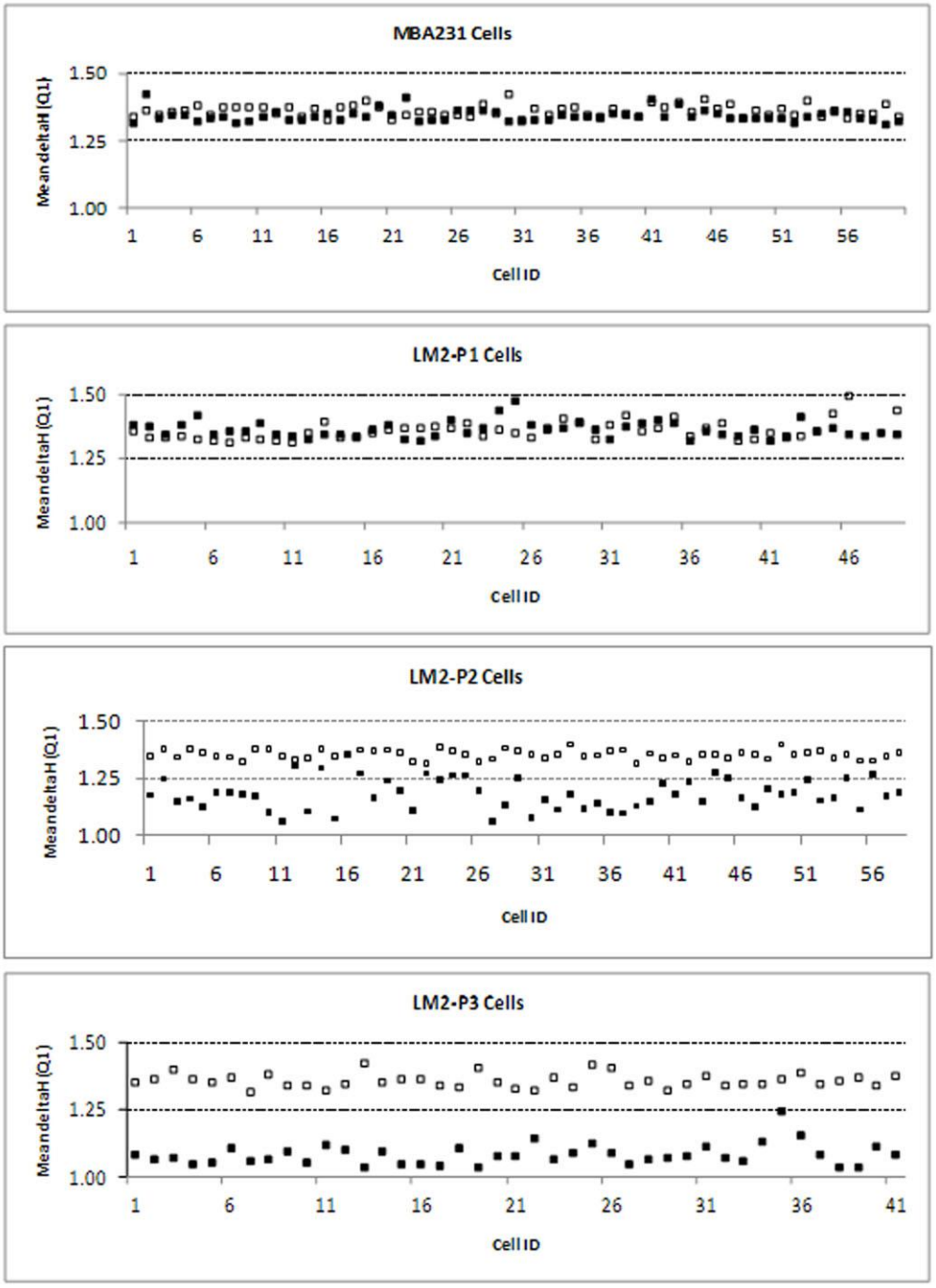

Figure 6. Lower quartile average single cell velocities of MDA-MB-231, LM2-P1, LM2-P2 and LM2-P3 cells on BSA and IL13Rα2-coated patches measured at the optimum assay conditions as described in Fig. 5 ΔH values from each MDA-MB-231 cell at 50 millisecond intervals are plotted to show relationships between individual cell velocities on BSA and IL13Rα2patches. Vertical axis represents the averaged 50 msec velocity. Horizontal axis is the identity of the cell. The two dots for each identity number belong to the same cell. Open squares are BSA and filled squares are anti-IL3RA2 velocities.

Task 3. To develop the use of the microfluidic device for *in vivo* tumor derived cells and define 99% sensitivity and specificity in identifying cells with lung metastasis capabilities from a tumor.

The goal of the microfluidic approach to diagnostic and prognostic assessment will be to be able to place a needle into a tumor in hard to reach places or in sequentially into tumors undergoing therapy to determine the frequency and characteristics of cell subpopulations. There are currently few available options to diagnostic assessment of tumors under these conditions. Since tumors are heterogeneous, multiple subpopulations will need to be assessed in multiple aspirates of different parts of the tumor. We conducted proof of principle studies by generating mixed tumors in mammary fat pads of NCr nu/nu mice under and IACUC protocol. We injected LM2 P3 Luc cells or MDA-MB-231 cells alone or mixed at ratios of 1:2, 1:9, 1:30 and 1:100. We imaged the mice in vivo using IVIS imaging (Figure 7). When tumors were 1 cm, we sacrificed the mice, extracted the tumors and needle aspirated them. Cells were analyzed by microfluidic technology. The remaining tumors were minced and single cell suspensions were generated and analyzed for IL13Rα2 by flow cytometry. Table 3 demonstrates close concordance between the percent cells positive of IL13Rα2 using the two assays. Flow cytometry does not account for tumor heterogeneity and averages cell content over the entire population. Multiple needle aspirates must be analyzed in the center and invasive edge of the tumor using microfluidics and compared with immunofluorescence to determine the true heterogeneity present.

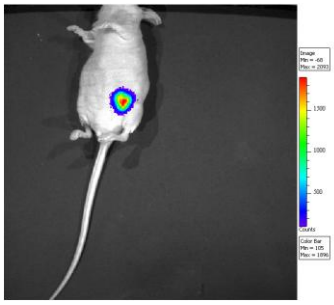

Figure 7. *In vivo* imaging of LM2 P3 xenograft tumor in NCr nu/nu mouse mammary fat pad.

Table 3. Comparable percentages of IL13Rα2 cells measured by microfluidics of needle aspirates from orthotopic xenograft tumors in NCr nu/nu mouse mammary fat pads (approximately 100 cells analyzed) and flow cytometry of dissociated tumors (approximately 1 million cells analyzed)

IL13Rα2+ Cells	100% LM2-P3 Tumor	1:2 Mixed Tumor	1:9 Mixed Tumor	1: 30 Mixed Tumor	1:100 Mixed Tumor	100% MDA-MB-231 Tumor
Microfluidics needle aspirated cells	**78%**	**30%**	**12%**	**3%**	**0%**	**0%**
Flow Cytometry dissociated tumor	**80%**	**21%**	**9%**	**4%**	**1%**	**0%**

4. Additional advances- Multiplexing

To characterize surface protein repertoires of individual cells in a tumor, the device has to be adapted for multiplexing capacity. We have successfully adapted the channel to print 7 patches with 6 different antibodies to six different proteins and analyzed several different cell lines for the expression of relevant antigens. Data have demonstrated that the expression of CXCR4, CD133, IL13Ra2 have prognostic significance in some tumor types, including tumor initiation, invasion, metastasis and treatment resistance in pancreatic cancer. Her2 and VCAM are important in breast cancer behavior and EPCAM is a general epithelial marker currently used to detect circulating tumor cells. We constructed a channel with seven 300 μm patches printed with antibodies to these

antigens and with one with BSA as a control and 100 μm spaces Figure 9 shows preliminary data with this multiplexed channel using a human pancreatic cell line PANC1. Figure 9A shows the design of the channel. The data demonstrate that four of the six antigens were expressed in some combination on 161 PANC1 cells assayed. A barcode for each cell is obtained for yes/no expression of each antigen depicted by the presence of slow velocities more than three standard deviation below the mean when the lower quartiles of measurements are analyzed. Figure 9B shows the velocity measurements of one of the cells which was positive for CD133, IL13Rα2, CXCR4 and EPCAM but only had rapid velocities on the patches coated with antibodies to Her2 and VCAM and with BSA. The frequency of cells for each of the 16 possible barcode combinations for the four positive antigens is depicted in Figure 9C. This demonstrates that multiplexing up to six antigens has been achieved successfully.

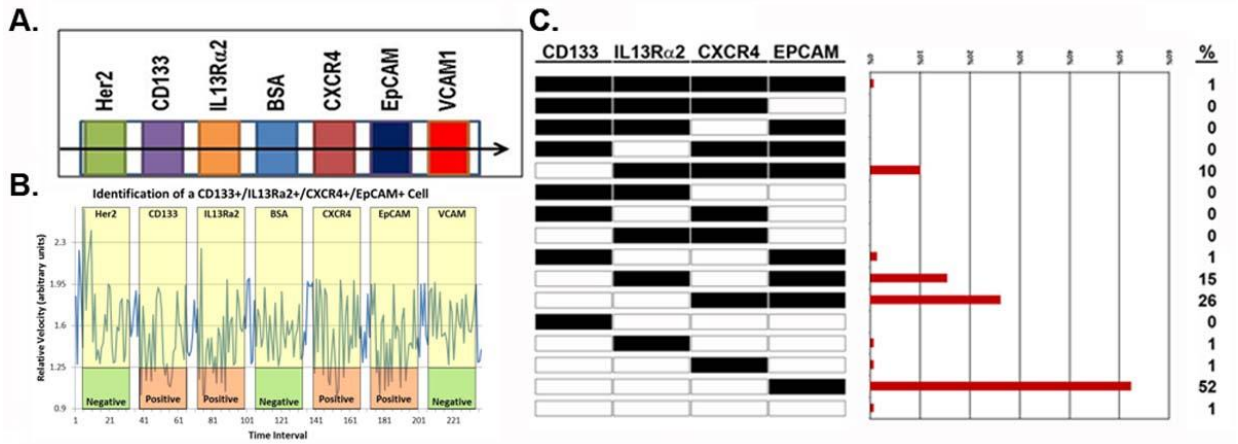

Figure 8. Multiplexing of six antigens relevant to a pancreatic cells lines's phenotype. Six patch channel was used to analyze PANC1 (and two other pancreatic cell lines to determine individual cell expression repertoire for six surface antigens reported significant in malignant, metastatic and tumor initiating potential

KEY RESEARCH ACCOMPLISHMENTS:

- Developed a biologically relevant model for studying flow retardation devices through the use of LM2 breast cancer cells with exclusive lung metastasizing capacity that had reliable and sustained cell surface expression of IL-13RA2.
- Developed a microfluidic device with
  - controlled fluid flow
  - cell introduction and collection capability
  - chamber dimension variability,
  - ligand patch distribution and dimension variability
  - antibody coating reliability
  - imaging capability needed for specific areas
  - data collection capability
  - data analysis capability
- Achieved 100% specificity and 100% sensitivity in identifying cells expressing surface protein on breast cancer cells only present on cells with exclusive lung metastatic potential using a novel, statistically and biophysically relevant identification model of incorporating only lower quartile measurements
- Achieved multiplexing with 6 surface antigens
- Was able to measure the presence of rare tumor cells in mixed cell tumors using needle aspirated cells with results comparable to flow cytometry.

REPORTABLE OUTCOMES:

The data has been presented at the 2011 and 2012 Annual American Association of Cancer Research Meeting (3, 7), the 2011 DOD Era of Hope meeting (4, 5), in a video prepared at the 2011 DOD Era of Hope meeting (6) and in a manuscript published in Lab on a Chip (8).

CONCLUSION:

We developed a microfluidic device with the capability to identify cells that express a surface protein present only on a breast cancer cell line capable of metastasizing exclusively to the lungs without the need tag the cells. The device detects the presence of the cell with a specific surface protein by slowing its rate of passage as it is pumped over a patch in the device coated with an antibody recognizing this protein. The rated of detection is 100% specific. These efforts will result in development of a device that will identify biologically significant cells in a sample derived by needle aspirates without having to subject them to labeling procedures that would result in their loss.

REFERENCES:

1. Minn AJ, Gupta GP, Siegel PM, Bos PD, Shu W, Giri DD, Viale A, Olshen AB, Gerald WL, Massague J. (2005) Genes that mediate breast cancer metastasis to lung. Nature 436:518-524.

2. Fitzpatrick E, McBride S, Yavelow J, Najmi S, Zanzucchi P and Wieder R. (2006) Microfluidic techniques for single cell protein expression analysis. Clin Chem 52:1080-1088.

3. Vellanki SH, Robin L. Smith RL, Tivari S, Kumar R, Wieder R. Development of a microfluidic device for tagless analysis of cell surface proteins directing lung metastasis. Proceedings of the American Association for Cancer Research 2011, v. 52, #5265, p. 1262.

4. Wieder R, Vellanki SH, Smith R, Kumar R. A microfluidic flow retardation approach for tagless assessment of individual breast cancer cell surface proteins mediating lung metastasis.The Department of Defense Breast Cancer Research Program Meeting, "Era of Hope". Orlando, FL, August 2011, #18-28 .

5. Rajan Kumar, Robin Smith, Sri Harikrishna Vellanki and Robert Wieder. Development and validation of a microfluidic device for tagless assessment of breast cancer cell surface proteins. The Department of Defense Breast Cancer Research Program Meeting, "Era of Hope". Orlando, FL, August 2011, #18-30

6. http://cdmrp.army.mil/pubs/video/bc/wieder_video.shtml

7. Sri HariKrishna Vellanki, Robin Smith, Rajan Kumar, Robert Wieder. Determination of single cell surface protein expression using a tagless microfluidic method. Proceedings of the American Association for Cancer Research 2012, v. 53, #4069, p 987.

8. Kumar R, Vellanki SH, Smith R and **Wieder R**. (2012) Determination of Single Cell Surface Protein Expression using a Tagless Microfluidic Method. Lab on a Chip 12:1646–1655.

APPENDICES:

None

SUPPORTING DATA:

All data appear in the body.